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A Transgenic *Drosophila melanogaster* Model To Study Human T-Lymphotropic Virus Oncoprotein Tax-1-Driven Transformation *In Vivo*

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Human T-cell lymphotropic virus type 1 (HTLV-1)-induced adult T-cell leukemia/lymphoma is an aggressive malignancy. HTLV-2 is genetically related to HTLV-1 but does not cause any malignant disease. HTLV-1 Tax transactivator (Tax-1) contributes to leukemogenesis via NF-κB. We describe transgenic *Drosophila* models expressing Tax in the compound eye and plasmatocytes. We demonstrate that Tax-1 but not Tax-2 induces ommatidial perturbation and increased plasmatocyte proliferation and that the eye phenotype is dependent on Kenny (IKKγ/NEMO), thus validating this new *in vivo* model.

Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy secondary to HTLV-1 (human T-cell lymphotropic virus type 1) infection (1). Although HTLV-1 and HTLV-2 are similar in genetic organization, they display major differences in pathogenesis and disease manifestation. HTLV-1 is capable of transforming T lymphocytes in infected individuals and subsequently leads to ATL, whereas HTLV-2 has not been clearly associated with malignant diseases but only with lymphocytosis (2, 3).

Transgenic mouse models overexpressing Tax-1 demonstrate its oncogenic properties (4–6). However, cellular pathways and *in vivo* Tax-1 partners that mediate Tax-1-induced cellular transformation are still unexplored. *Drosophila melanogaster* is a valuable model because of the availability of facile genetic screens, a nearly complete collection of mutants, RNA interference (RNAi) lines, and advanced genetic technologies, in addition to highly conserved pathways such as NF-κB. Acquisition of a "rough-eye" phenotype is an accepted surrogate for cell transformation in the *Drosophila* model. Using *Drosophila* transgenic models expressing Tax proteins (Tax-1 and Tax-2), we present evidence demonstrating the ability of Tax-1 but not Tax-2 to induce transformation in *Drosophila* and show that this transformation is dependent primarily on Kenny, the *Drosophila* orthologue of IKKγ/NEMO, upstream of Relish (NF-κB) activation.

Briefly, we overexpressed Tax-1 specifically in developing imaginal eyes by using the glass multimer reporter promoter GMR-Gal4 (number 9146; Bloomington Drosophila Stock Center, NIH P40OD018537). Tax-1 was amplified from pSG5M-Tax (7) and cloned into the *Drosophila* expression vector pUAST (GenScript) with an N-terminal Myc tag. Plasmid pUAST-Tax-1 was used for the generation of transgenic fly strains after injection into wild-type white-eyed (*white*$^-$) embryos (BestGene). Successful transgenesis was monitored through the appearance of the red-eye phenotype (*white*$^+$). Analysis of the ommatidial structure by scanning electron microscopy was performed with a Tescan Mira3 LMU field emission gun scanning electron microscope. A grading system based on the severity of the eye phenotype (number of ommatidial fusions and extent of bristle organization) was developed, and statistical tests were done by one-way analysis of variance. While control flies displayed normal eyes (Fig. 1A, left panel), the eyes of adult flies expressing a single copy of Tax-1 under the control of GMR-GAL4 showed a rough-eye phenotype, which appeared as a perturbation of the normal crystalline array of the ommatidia, with fused ommatidial structures, as well as lost and duplicated bristles in some instances (Fig. 1A, compare left and middle panels, i.e., GMR versus GMR>UAS-Tax-1; see panel B for quantification). Expression of two copies of the Tax-1 transgene (Fig. 1A, right panel, homozygous) significantly enhanced the eye phenotype (see Fig. 1B for quantification). Tax-1 expression was confirmed in whole protein extracts by Western blotting with a mouse monoclonal antibody against Myc (9E10, a kind gift from Bengt Hallberg) and a rabbit polyclonal antibody against β-actin (A2066; Sigma-Aldrich) (Fig. 1C).

Since HTLV-1 specifically induces lymphocyte transformation in humans, we next overexpressed Tax-1 in hemocytes, i.e., *Drosophila* blood leukocyte-like cells, as plasmatocytes account for 95% of the circulating hemocytes in *Drosophila* larvae (reviewed in reference 8). UAS-Tax-1 flies were crossed to flies expressing GAL4 under the plasmatocyte-specific *peroxidasin* promoter

Received 8 April 2015 **Accepted** 14 May 2015
Accepted manuscript posted online 20 May 2015
Citation Shirinian M, Kambris Z, Hamadeh L, Grabbe C, Journo C, Mahieux R, Bazarbachi A. 2015. A transgenic *Drosophila melanogaster* model to study human T-lymphotropic virus oncoprotein Tax-1-driven transformation *in vivo*. J Virol 89:8092–8095. doi:10.1128/JVI.00918-15.
Editor: S. R Ross
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doi:10.1128/JVI.00918-15

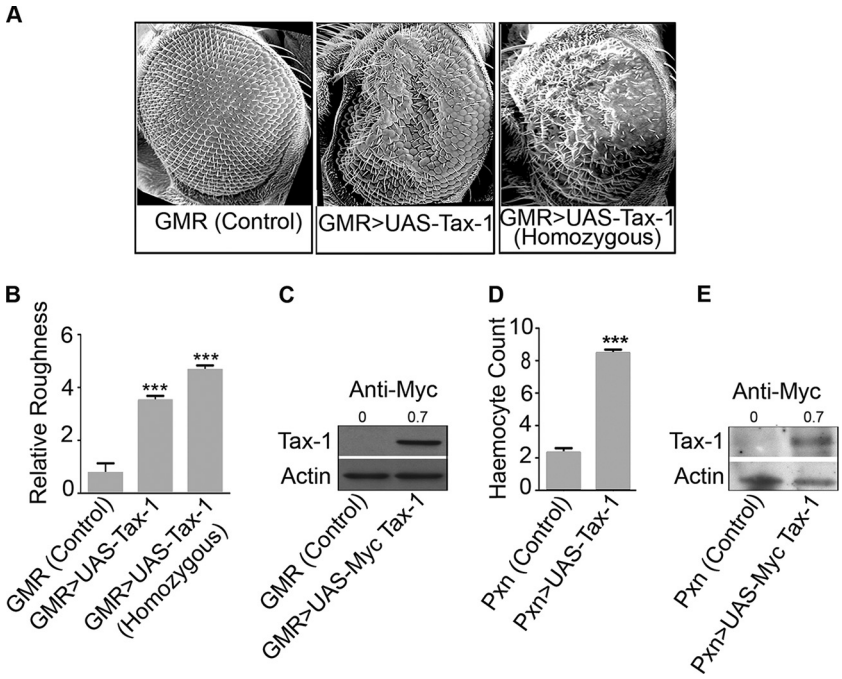

FIG 1 Overexpression of Tax-1 induces transformation in *Drosophila*. (A) Representative scanning electron microscopy images of adult eyes of transgenic flies expressing the genotypes indicated under the control of the eye-specific GMR promoter (GMR-GAL4). All of the flies contain one copy of the transgene, unless otherwise stated. Homozygous flies contain two copies of the transgene. GMR flies are controls with no UAS transgene. (B) Relative roughness was quantified on the basis of the number of ommatidial fusions and the extent of bristle organization. ***, $P < 0.001$. (C) Cell lysates (150 μg) from control and UAS-Tax1 transgenic adult whole flies were subjected to electrophoresis and probed with an anti-Myc or anti-actin antibody. (D) Hemocyte counts in transgenic larvae expressing transgenic Tax-1 under the control of the plasmatocyte-specific *peroxidasin* promoter (Pxn-GAL4). ***, $P < 0.001$. (E) Cell lysates (150 μg) from UAS-Tax-1 transgenic larvae were analyzed by Western blotting, confirming the expression of the UAS-Tax-1 transgene in larval hemocytes.

(Pxn-GAL4) (9). Third-instar larvae were bled, and hemocytes were loaded onto a Neubauer hemocytometer for counting. A robust and statistically significant increase ($P < 0.001$) in hemocyte numbers, compared to those of control larvae, was observed upon Tax-1 overexpression (Fig. 1D). Transgene expression in hemocytes was verified by Western blotting (Fig. 1E). This finding demonstrates that ectopic Tax-1 expression induces an overgrowth of leukocyte-like cells in *Drosophila* and further strengthens a transforming activity of Tax-1 in this model.

To further show that *Drosophila* is a relevant model to genetically characterize the mechanisms of Tax-1-induced cell transformation, we took advantage of the rough-eye phenotype induced by Tax-1 to screen for genes interacting with Tax-1. Previous reports have suggested that NF-κB activation plays a key role in Tax-induced transformation (reviewed in reference 10). In *Drosophila*, the NF-κB family member Relish is activated following IMD (immune deficiency) pathway activation, which is dependent on Kenny (the *Drosophila* orthologue of IKKγ/NEMO). In parallel, Dorsal and DIF are activated following engagement of the Toll receptor (e.g., the Toll pathway), independently of Kenny (reviewed in reference 11). Together, the IMD and Toll pathways induce the expression of multiple antibacterial and antifungal peptides, including diptericin, drosomycin, cecropin, and defensin. *Drosophila* transgenic UAS-RNAi lines (*Kenny*, VDRC GD1249 and KK107280; *Dorsal*, VDRC GD1238; *Relish*, VDRC GD1199) were crossed to GMR-Gal4 to induce a knockdown of the target genes specifically in the compound eye (UAS-GAL4 system). The efficiency of inhibition induced by RNAi was validated by quantitative reverse transcription (RT)-PCR (data not shown). RNAi-mediated silencing of Relish significantly reduced the rough-eye phenotype induced by Tax-1 ($P < 0.001$) (Fig. 2A, second pair of images from the top; see panel B for quantification), whereas reduction of Dorsal modulated the rough-eye phenotype induced by Tax-1 to a lesser extent ($P < 0.01$) (Fig. 2A, third pair of images from the top; see panel B for quantification). This suggests that Tax-1-driven cell transformation specifically requires Relish activation and, by extension, that it is dependent primarily on the IMD pathway. To confirm that Tax-1 activates the IMD pathway in *Drosophila*, we investigated whether Relish-dependent transcription is affected in Tax-1-expressing flies by using *diptericin* expression levels as a readout. Quantitative RT-PCR indeed showed that *diptericin* expression was higher in flies expressing Tax-1 than in control flies (Fig. 2C). Importantly, *diptericin* expression was not increased in Relish-silenced Tax-1-transgenic flies (Fig. 2C), confirming that Relish is a major inducer of Diptericin downstream of Tax-1.

Tax-1 binds to IKKγ/NEMO (reviewed in reference 10). Since Kenny, the *Drosophila* orthologue of IKKγ/NEMO, is specifically involved in Relish activation, we hypothesized that the transforming activity of Tax-1 in the *Drosophila* compound eye could be caused by an interaction between Tax-1 and Kenny. In agreement, RNAi-mediated silencing of *kenny* strongly reduced the rough-eye phenotype induced by Tax-1 expression ($P < 0.001$) (Fig. 2A, fourth pair of images from the top; see panel B for quantification), indicating that Kenny is required for Tax-1-driven cell transformation in *Drosophila*.

Given that the genetically related virus HTLV-2 does not cause cell transformation in infected individuals, we generated trans-

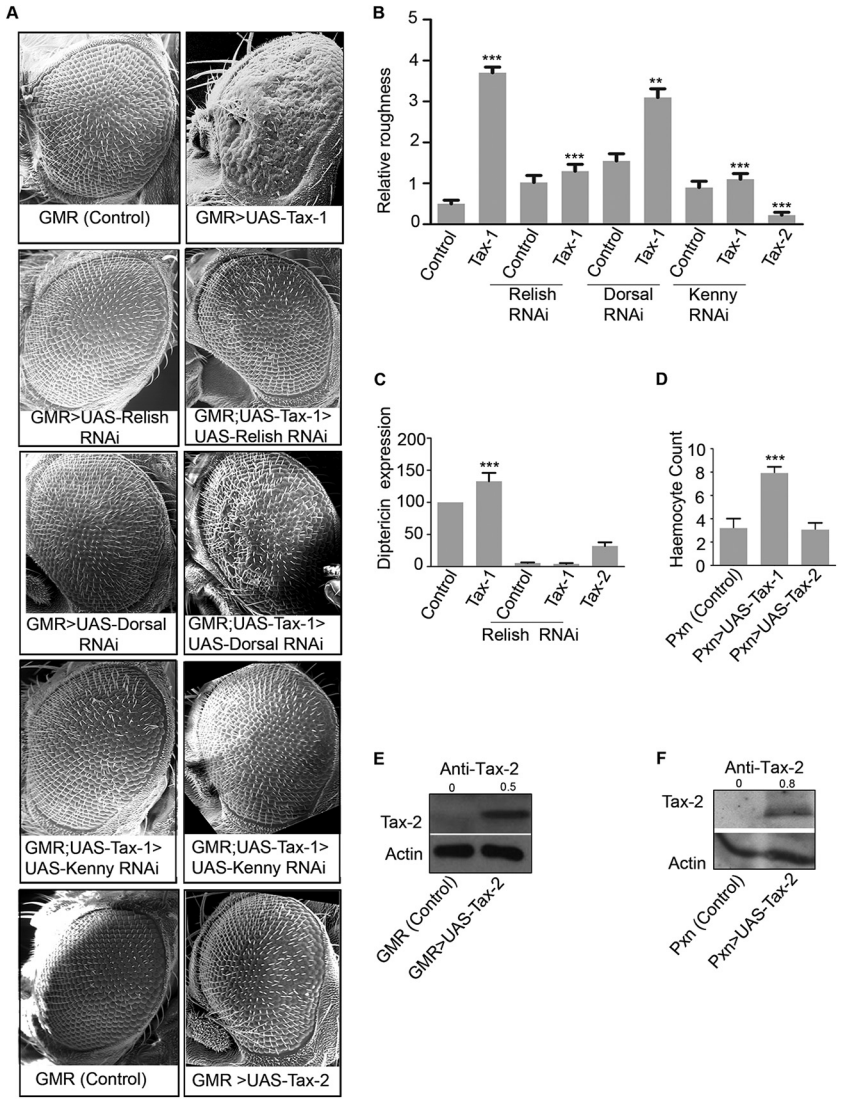

FIG 2 NF-κB pathway components Kenny and Relish are necessary for Tax-1-induced transformation in *Drosophila*, and Tax-2 does not activate the Relish pathway or cause cell transformation. (A) Relish, Dorsal, and Kenny expression was inhibited by RNAi in flies overexpressing Tax-1 in the compound eye. Representative scanning electron microscopy micrographs of adult eyes are shown. Quantification of the relative roughness of the compound eye of each fly strain is shown in panel B. ***, $P < 0.001$; **, $P < 0.01$. (C) Levels of expression of *diptericin*, a Relish target gene encoding an antimicrobial peptide, in the transgenic flies indicated. ***, $P < 0.001$. (D) Hemocyte counts in transgenic larvae expressing Tax-1 and Tax-2 under the control of the plasmatocyte-specific *peroxidasin* (Pxn) promoter. ***, $P < 0.001$. (E) Cell lysates (150 μg) from transgenic whole adult flies were analyzed by Western blotting, confirming Tax-2 expression in the transgenic flies. (F) Lysates (150 μg) of hemocytes from Tax-2 transgenic larvae were analyzed by Western blotting, confirming expression of the Tax-2 transgene.

genic flies expressing HTLV-2 Tax (Tax-2) to enable comparative studies of Tax-1 and Tax-2. The 6×His-tagged UAS-Tax-2 transgene was expressed in the compound eye or in hemocytes. We first found that, in contrast to those of Tax-1-expressing flies, the compound eyes of Tax-2-expressing flies (GMR>UAS-Tax-2) displayed ommatidia that were arranged in a normal crystalline structure (Fig. 2A, bottom pair of images; see panel B for quantification). Tax-2 expression was verified by Western blotting with a rabbit polyclonal antibody against Tax-2 (12) (Fig. 2E). Consistent with these results, the expression of *diptericin* was not upregulated in response to ectopic Tax-2 expression (Fig. 2C), indicating that Tax-2 is a poor activator of Relish. Thus, the inability of Tax-2 to induce cell transformation in *Drosophila* is correlated with an absence of Relish target gene activation. Finally, the number of hemocytes was not increased in Tax-2 expressing flies (Fig. 2D; see panel F for analysis of Tax-2 expression by Western blotting).

In this study, we have generated transgenic *Drosophila* expressing the HTLV-1 oncoprotein Tax-1 or its HTLV-2 (Tax-2) counterpart. This system is particularly appropriate since cell transformation in the developing eye results in an easily screenable rough-eye phenotype in adult flies. This *in vivo* model is validated by the demonstration that the NF-κB pathway is required for Tax-1-driven cell transformation and that Tax-2 fails to induce cell transformation, consistent with epidemiological and experimental data (3). This model will be of great importance because it will allow rapid screening of a series of Tax-1 mutants that are im-

paired for NF-κB activation, CREB activation, and SRF activation (13); lack the ability to bind CBP/p300 (14) or p/CAF (15); and lack nuclear localization (16), posttranslational modifications (10), a PDZ-binding motif, etc., as well as a series of candidate cellular genes possibly linked to Tax-1 transforming activity. Transgenic *Drosophila* is therefore an important new tool for deciphering how HTLV-1 Tax transforms cells *in vivo*.

ACKNOWLEDGMENTS

This study was supported by the Swedish Research Council, the American University of Beirut Medical Practice Plan, the University Research Board, and the Lebanese National Council for Scientific Research. M.S. is supported by the Lady TATA memorial trust and the Swedish Research Council. C.G. is supported by the Swedish Research Council. Z.K. is supported by the American University of Beirut (AUB) University Research Board (award 102725). C.J. and R.M. are supported by the Ecole Normale Supérieure de Lyon. R.M. was also supported by a Contrat Hospitalier de Recherche Translationnelle. R.M. and C.J. acknowledge the support of ARC and of La Ligue Contre le Cancer (Program Équipe Labelisée).

We thank CRSL (AUB Central Research Science Laboratory) for their help in scanning electron microscopy imaging.

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